

Integration of low temperature and light signaling during cold acclimation response in *Arabidopsis*

Rafael Catalá, Joaquín Medina¹, and Julio Salinas²

Departamento de Biología Medioambiental, Centro de Investigaciones Biológicas-Consejo Superior de Investigaciones Científicas, Ramiro de Maeztu, 9, 28040 Madrid, Spain

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Certain plants increase their freezing tolerance in response to low nonfreezing temperatures, an adaptive process named cold acclimation. Light has been shown to be required for full cold acclimation, although how light and cold signals integrate and cross-talk to enhance freezing tolerance still remains poorly understood. Here, we show that HY5 levels are regulated by low temperature transcriptionally, via a CBF- and ABA-independent pathway, and posttranslationally, via protein stabilization through nuclear depletion of COP1. Furthermore, we demonstrate that HY5 positively regulates cold-induced gene expression through the Z-box and other cis-acting elements, ensuring the complete development of cold acclimation. These findings uncover unexpected functions for HY5, COP1, and the Z-box in *Arabidopsis* response to low temperature, provide insights on how cold and light signals integrate to optimize plant survival under freezing temperatures, and reveal the complexity of the molecular mechanisms plants have evolved to respond and adapt to their fluctuating natural environment.

Plants have evolved a variety of adaptive mechanisms to survive adverse environmental conditions. In the case of freezing temperatures, which negatively affect plant growth and distribution, and affect crop quality and productivity, some species are able to increase their tolerance after low-nonfreezing temperature exposure, an adaptive process termed cold acclimation (1). External signals, however, cannot be considered in isolation when studying the adaptive responses plants have evolved to survive in an ever-changing environment. Plants must process and integrate the surrounding signals to adequately respond to changes in their environmental conditions. The correct integration of low temperature and light signals, for instance, is crucial to ensure the appropriate development of cold acclimation. Thus, light is required for the increase in freezing tolerance that is produced during cold acclimation in *Arabidopsis* (2). The role of light in cold acclimation seems to be mediated through the phytochromes (3) and would consist in positively regulating cold-induced gene expression (4, 5). Consistent with these results, expression analysis in *Arabidopsis* have revealed that light is required for cold induction of several genes involved in cold acclimation, including CBFs (5, 6). Interestingly, light quality has also been described to have a function in regulating plant freezing tolerance. A low red to far-red ratio light signal increases CBF expression in *Arabidopsis*, this increase being sufficient to confer freezing tolerance at temperatures higher than those required for cold acclimation (7). All these studies evidence a complex cross-talk between light and low temperature signals in the regulation of cold acclimation. The pathways and molecular components that mediate such a cross-talk, however, still remain largely unknown.

HY5 is an *Arabidopsis* bZIP transcription factor that has a pivotal role in light signaling, mediating photoreceptor responses to promote photomorphogenesis (8). In addition, it has also been described to mediate plant responses to UV-B (9) and to different hormones, such as ABA, gibberellins, cytokinin, and auxins (8). Recently, a ChIP-chip approach has revealed that HY5 recognizes several light-responsive elements, including the Z-box, and binds >9,000 genes, detectably affecting the expression of >1,100 targets (10). Further, HY5 indirectly regulates many other genes

through subnetworks mediated by other regulators (10). Therefore, HY5 seems to be one of the central modulators of gene expression for the coordination of light signals and plant development. Consistent with this relevant function, HY5 levels are strongly regulated. At the transcriptional level, HY5 expression is positively regulated by light via a phytochrome-dependent pathway (11). Posttranslationally, HY5 is regulated by the E3 ubiquitin ligase COP1, a crucial repressor of light signaling. In the dark, it is turned over in the nucleus by COP1 (12). In the light, COP1 is excluded from the nucleus, allowing HY5 increase stabilization and activation of light-responsive genes (12). COP1 has also been shown to be depleted from the nucleus in response to gibberellins and cytokinin (8). Intriguingly, however, although COP1 is not excluded from the nucleus in response to UV-B, HY5 is not degraded (9).

We reported that the expression of *CAB1*, an *Arabidopsis* light-regulated gene, is induced by cold, indicating that *CAB1* constitutes a common intermediate of *Arabidopsis* responses to light and low temperature (13). To further understand the complex integration of cold and light signaling, we have investigated the molecular mechanisms underlying the cold induction of *CAB1*. Here, we show that the induction of *CAB1* in response to low temperature is mediated by HY5 through the Z-box, which constitutes a low temperature responsive element (LTRE). Our results demonstrate that, in addition to *CAB1*, HY5 mediates the induction of ≈10% of all *Arabidopsis* cold-inducible genes, including those involved in anthocyanin biosynthesis, ensuring the complete development of cold acclimation. Interestingly, we also demonstrate that HY5 levels are regulated by low temperature transcriptionally, via a CBF- and ABA-independent pathway, and posttranslationally, via protein stabilization through the nuclear depletion of COP1. These data indicate that HY5, COP1, and the Z-box integrate cold and light signaling to promote the cold acclimation response.

Results

Z-Box Is an LTRE That Mediates cold Induction of *CAB1*. We showed that the expression of *Arabidopsis CAB1* gene is positively regulated by low temperature at the transcriptional level (13). The *CAB1* promoter does not contain any cis-acting element implicated in cold-regulated gene expression, indicating that an unknown LTRE should mediate the cold response of *CAB1*. To identify this element, constructs containing different regions of the *CAB1* promoter transcriptionally fused to the *GUS* gene

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¹Present address: Departamento de Biotecnología INIA, Centro de Biotecnología y Genómica de Plantas, Campus de Montegancedo, 28223 Madrid, Spain.

²To whom correspondence should be addressed. E-mail: salinas@cib.csic.es.

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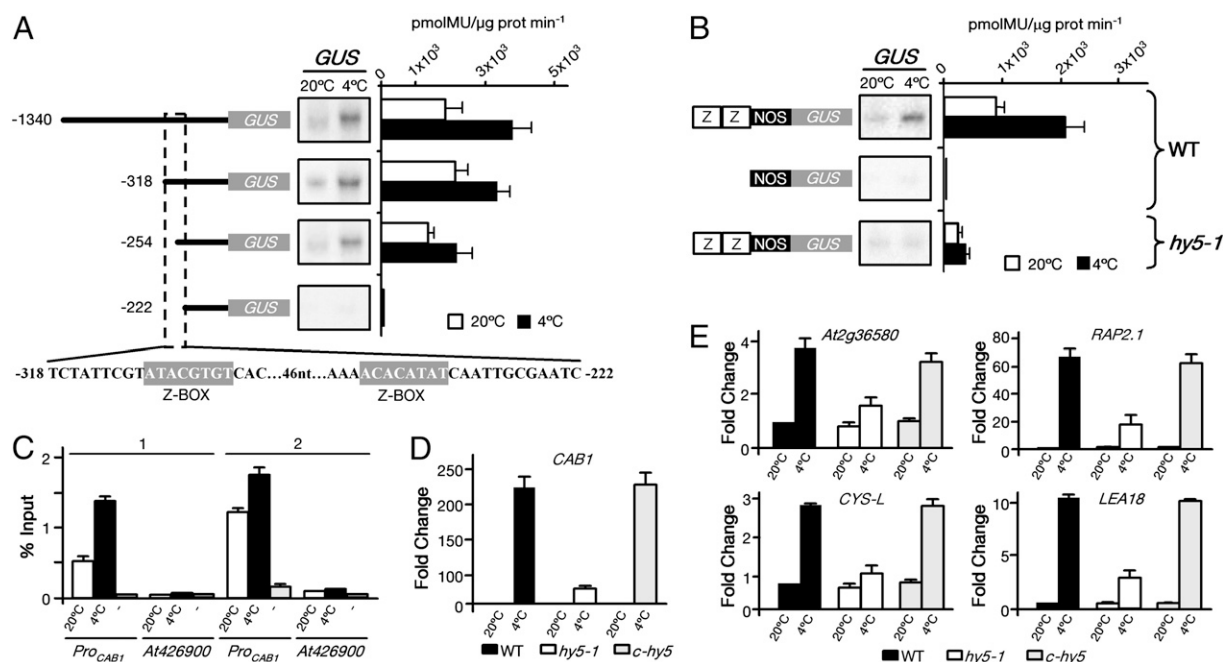


Fig. 1. HY5 activates cold-induced gene expression through the Z-box. *GUS* expression and activity in WT etiolated seedlings containing different *CAB1* promoter:*GUS* fusions (A) or the *Z:NOS:GUS* and *NOS:GUS* fusions (B), and in *hy5-1* etiolated seedlings containing the *Z:NOS:GUS* fusion (B) grown at 20 °C or exposed 24 h to 4 °C. Data from *GUS* activity are expressed as means of three independent experiments with 10 plants each. Bars indicate SD. The two Z-box elements included in the -318/-222 *CAB1* promoter fragment are displayed in A. (C) ChIP of DNA associated with HY5:YFP expressed under the control of its own promoter in *c-hy5* etiolated seedlings growth at 20 °C or exposed 24 h to 4 °C. ChIP-qPCR was performed with an anti-GFP antibody for the -318/-222 *CAB1* promoter fragment (*ProCAB1*) and an intergenic control region between genes *At4g26900* and *At4g26910* (At4g26900). A ChIP-qPCR assay in cold treated seedlings without anti-GFP antibody was also included as a control (-). Data are from two independent biological replicates (1, 2) and are presented as the percentage recovered from the total input DNA before immunoprecipitation (% input). (D) Expression analysis of *CAB1* determined by qPCR in WT, *hy5-1*, and *c-hy5* etiolated seedlings grown at 20 °C or exposed 24 h to 4 °C. (E) Expression analysis of *At2g36580*, *RAP2.1*, *CYS-L*, and *LEA18* genes determined by qPCR in WT, *hy5-1*, and *c-hy5* plants grown at 20 °C or exposed 24 h to 4 °C. In D and E, data were normalized to the expression levels of the control gene *At4g26410*. In C-E, bars indicate SD of triplicates.

(Fig. 1A) were introduced into *Arabidopsis*, and *GUS* expression and activity were analyzed in 4-d-old etiolated seedlings grown under control conditions or exposed to 4 °C. Cold induction was restricted to transgenic lines with constructs including at least one of the two Z-boxes that harbors the 1,340-bp *CAB1* promoter, the fragment of 96 bp containing the two Z-boxes being necessary for full induction (Fig. 1A). These results suggested that the Z-box, a motif involved in light-regulated gene expression (14), could be responsible for the cold induction of *CAB1*. To confirm this assumption, 4-d-old *Arabidopsis* etiolated seedlings containing a dimer of the Z-box fused to the NOS basal promoter and to the *GUS* gene (*Z:NOS:GUS*) (15) were examined for their *GUS* expression and activity in response to low temperature. Fig. 1B shows that the Z-box dimer confers similar levels of relative cold induction than the 1,340-bp *CAB1* promoter. Thus, the Z-box constitutes the LTRE that mediates the cold induction of *CAB1*.

HY5 Regulates Low Temperature-Induced Gene Expression. Because the Z-box motif mediates the induction of *CAB1* by light through HY5 (14), we investigated whether HY5 was also required for the low temperature-induced gene expression mediated by the Z-box. We analyzed *GUS* expression and activity in 4-d-old wild type (WT) and *hy5-1* etiolated seedlings containing the *Z:NOS:GUS* construct (14) grown at 20 °C or exposed to 4 °C. Both values were severely reduced in cold-exposed *hy5-1* seedlings (Fig. 1B), revealing that HY5 is needed for cold-induced gene expression through the Z-box. Moreover, chromatin immunoprecipitation (ChIP)-quantitative PCR (qPCR) assays using *hy5-1* mutant plants complemented with a *ProHY5:HY5:YFP* construct (*c-hy5*) (9) (Fig. S1) showed that HY5 directly binds to the 96-bp

CAB1 promoter fragment containing the two Z-boxes and that this binding is enriched under cold conditions (Fig. 1C). Consistently, the induction of *CAB1* expression in response to low temperature was impaired in *hy5-1* seedlings (Fig. 1D). *c-hy5* plants exhibited a WT expression pattern of *CAB1* (Fig. 1D), confirming that the decreased induction of *CAB1* in *hy5-1* was due to the absence of HY5.

The results described above prompted us to examine the significance of HY5 in mediating cold-induced gene expression. Transcript profiling of 3-wk-old *hy5-1* plants exposed 1 d at 4 °C allowed the identification of 426 genes whose expression levels were reduced at least twofold compared with its *Ler* WT ecotype (Dataset S1). Remarkably, 103 of these genes are induced in response to low temperature (16) (Table S1), which represents ≈10% of all *Arabidopsis* cold-inducible genes (17). Moreover, 56 of the 103 genes are not induced by light (16), indicating that the sub-regulons controlled by HY5 in response to light and low temperature are not completely coincident (Table S1). The microarray results were validated analyzing the expression of several cold-inducible genes in *Ler*, *hy5-1*, and *c-hy5* plants by qPCR (Fig. 1E). Altogether, these data provide evidence that HY5 has an important function in regulating cold-induced gene expression. Several HY5-regulated cold-inducible genes, including those validated by qPCR, are related to cold acclimation (18–21), suggesting that HY5 may also have a role in this adaptive response.

HY5 Is a Positive Regulator of Cold Acclimation. The findings reported before led us to analyze the freezing tolerance of *hy5-1* mutant before and after being cold acclimated (7 d; 4 °C). Non-acclimated *hy5-1* plants displayed a similar capacity to tolerate

freezing temperatures as the *Ler* ecotype, the LT_{50} (temperature that causes 50% lethality) values being in both cases approximately -5°C (Fig. S2A and B). In contrast, *hy5-1* plants exhibited significant lower freezing tolerance than *Ler* plants after 1 wk of cold acclimation, the LT_{50} values being in this case -7.7°C and -8.5°C , respectively (Fig. 2A and B). Cold-acclimated *c-hy5* plants recovered the freezing tolerance of WT plants (Fig. 2A and B), demonstrating that the null mutation *hy5-1* (22) provokes a significant reduction in the capacity of *Arabidopsis* to cold acclimate and, therefore, that HY5 is a positive regulator of this adaptive response.

HY5 Controls Anthocyanin and ROS Accumulation. During cold acclimation, *hy5-1* plants manifested lower accumulation of anthocyanins than *Ler* (Fig. 3A and B). Consistent with this observation, the expression levels of cold-inducible genes *CHALCONE SYNTHASE* (*CHS*), *CHALCONE ISOMERASE* (*CHI*), and *FLAVONOL SYNTHASE* (*FLS*), which encode critical enzymes in the anthocyanin biosynthetic pathway (23), were clearly affected in cold-treated *hy5-1* mutants compared with *Ler* (Fig. 3C). Anthocyanins protect photosystems from photoinhibition, avoiding the accumulation of high levels of reactive oxygen species (ROS) when plants are subjected to different abiotic stresses, including low temperature (24). Then, it was predictable that *hy5-1* mutants exposed to low temperature have elevated levels of ROS, which, considering their negative effect on cold acclimation (25, 26), would account for their impaired capacity to cold acclimate. Results showed that ROS levels in cold-treated *hy5-1* mutants, quantified with 2,7-dichlorodihydrofluorescein diacetate (DCFH₂-DA) or visualized with Nitroblue Tetrazolium (NBT), are much higher than in *Ler* (Fig. 3D and E). The analysis of *c-hy5* plants confirmed that all mutant phenotypes observed were caused by the *hy5-1* mutation (Fig. 3). HY5, therefore, activates cold-induced anthocyanin accumulation, which is essential to prevent high levels of ROS and to ensure the complete development of cold acclimation response. According to this role of anthocyanin accumulation in cold acclimation, *Arabidopsis* mutants for *CHS* (*tt4*) and double mutants for *CHS* and *CHI*

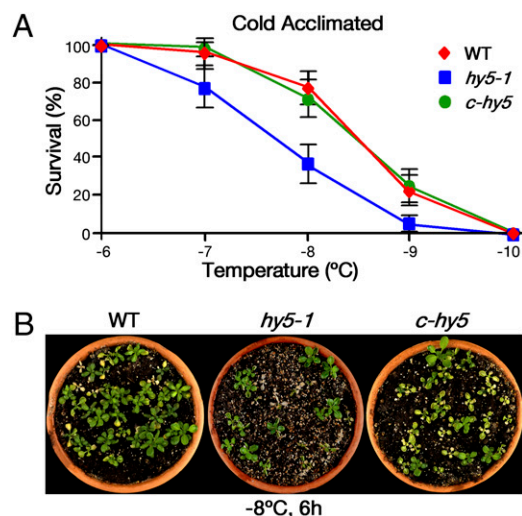


Fig. 2. HY5 positively regulates cold acclimation. Two-week-old WT, *hy5-1*, and *c-hy5* plants were exposed to the indicated freezing temperatures for 6 h after being acclimated 7 d at 4°C . Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 7 d of recovery under control conditions. Data are expressed as means of three independent experiments with 50 plants each. Bars indicate SD. (A) Freezing tolerance of cold-acclimated WT, *hy5-1*, and *c-hy5*. (B) Representative cold-acclimated plants 7 d after being exposed to -8°C for 6 h.

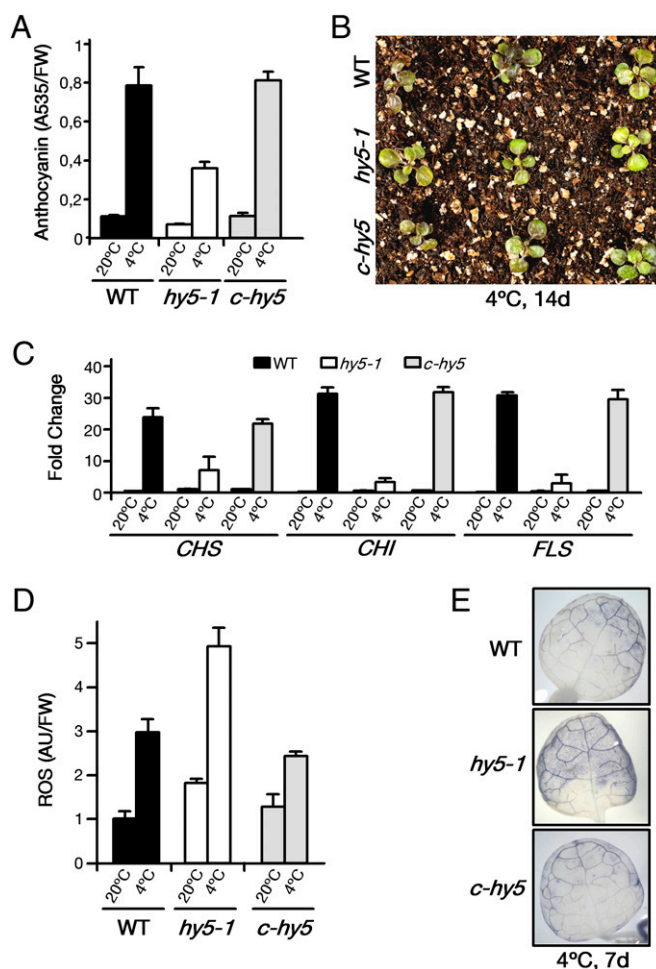


Fig. 3. HY5 promotes anthocyanin biosynthesis and restrain ROS accumulation in response to low temperature. (A) Anthocyanin levels in WT, *hy5-1*, and *c-hy5* plants grown at 20°C or exposed 7 d to 4°C . (B) Representative WT, *hy5-1*, and *c-hy5* plants exposed to 4°C . (C) Expression analysis of *CHI*, *CHS*, and *FLS* genes determined by qPCR in WT, *hy5-1*, and *c-hy5* plants grown at 20°C or exposed 24 h to 4°C . Data were normalized to the expression levels of the control gene *At4g26410*. Bars indicate SD of triplicates. (D) ROS levels quantified with DCFH₂-DA in WT, *hy5-1*, and *c-hy5* plants exposed 7 d to 4°C . AU, arbitrary units; FW, fresh weight. (E) ROS levels in representative leaves of WT, *hy5-1*, and *c-hy5* plants exposed to 4°C and stained with NBT. In A and D, data are expressed as means of three independent experiments with 25 and 10 plants each, respectively. Bars indicate SD.

(*tt4tt5*), which are affected in anthocyanin biosynthesis and accumulate high levels of ROS (24), exhibit a reduced capacity to cold acclimate (Fig. S3).

HY5 Expression Is Transcriptionally Regulated by Low Temperature Through a CBF- and ABA-Independent Pathway. To further understand the function of HY5 in cold acclimation, we explored the possibility that the *HY5* gene could be subjected to low temperature regulation. *HY5* transcripts accumulated transiently in plants exposed to 4°C under long day conditions, reaching a peak after 3 h of treatment (Fig. 4A). In etiolated plants, *HY5* transcripts also accumulated when exposed to 4°C in the dark although to a lesser extent (Fig. 4A). The accumulation of *HY5* mRNAs in response to low temperature was not affected in ABA- (*aba2*) and CBF- (*cbf2* and *CBF1-AS3*) deficient plants, indicating that *HY5* is induced by low temperature through an ABA- and CBF-independent pathway (Fig. 4B). Analysis of *Arabidopsis*

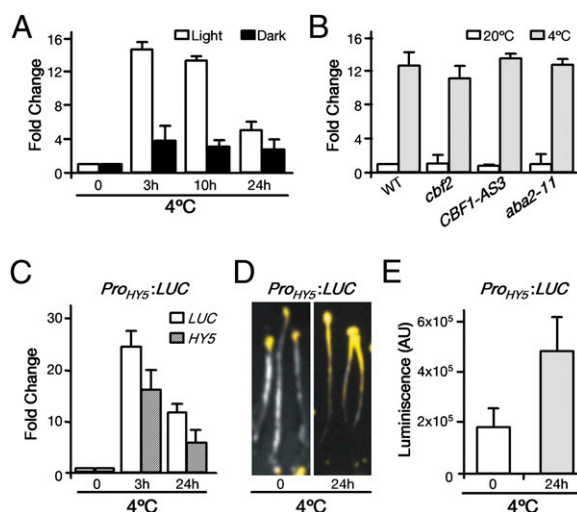


Fig. 4. The expression of *HY5* is regulated at the transcriptional level by low temperature independently of ABA and CBFs. Expression analysis of *HY5* determined by qPCR in Col plants exposed to 4 °C in the light or in the dark (A), and in cold-exposed WT, *cbf2* mutant, *CBF1-AS3* transgenic, and *aba2-11* mutant plants (B). (C) Expression analysis of *LUC* and *HY5* genes determined by qPCR in *Pro_{HY5}:LUC* plants exposed to 4 °C at the indicated times. In A–C, data were normalized to the expression levels of the control gene *At4g26410*. Bars indicate SD of triplicates. (D) LUC activity in etiolated *Pro_{HY5}:LUC* seedlings exposed 24 h to 4 °C. (E) Quantification of LUC activity shown in D. Data are expressed in arbitrary units as means of three independent experiments with 10 plants each. Bars indicate SD.

transgenic lines containing the reporter *LUC* gene fused to a 1,500-bp *HY5* promoter fragment (*Pro_{HY5}:LUC*) (27) revealed that both *LUC* expression (Fig. 4C) and activity (Fig. 4D and E) increased by low temperature, indicating that the cold induction of *HY5* is regulated at the transcriptional level.

Low Temperature Induces *HY5* Stabilization Through Nuclear Exclusion of COP1. *HY5* has been shown to be stabilized by light (12). Hence, we obtained *hy5-1* mutant plants complemented with a *35S:HY5:3HA* construct (Fig. S4) to investigate whether *HY5* might also be posttranslationally regulated by low temperature through protein stabilization. As expected from previous work (12), the *HY5:3HA* protein was clearly degraded in the dark (8 h) and stabilized under light conditions in plants grown at 20 °C (Fig. 5A). Interestingly, however, *HY5:3HA* was not degraded after 8 h in the dark at 4 °C (Fig. 5A), suggesting that low temperature stabilizes *HY5*. This result was confirmed by confocal microscopy analysis in *c-hy5* plants. We found high levels of *HY5:YFP* protein in the nucleus of transgenic lines grown under light conditions at 20 °C or in the dark at 4 °C, but not in transgenic plants grown in the dark at 20 °C (Fig. 5B). Under dark conditions, *HY5* is a substrate of the E3 ubiquitin ligase COP1 in the nucleus, which leads to its ubiquitination and subsequent proteasomal degradation (12). Light exposure, in turn, induces nuclear depletion of COP1, allowing *HY5* stabilization (12). Therefore, we intended to determine the subcellular localization of COP1 in the dark at 4 °C, when high levels of *HY5* are present in the nucleus. Using *cop1-4* mutant plants complemented with a *Pro_{35S}:YFP:COP1* fusion (9), we observed that, conforming to earlier data (12), the YFP:COP1 protein localized in the nucleus of plants grown in the dark but not in the nucleus of plants grown in the light at 20 °C (Fig. 5C). Remarkably, complemented *cop1-4* plants grown in the dark at 4 °C did not display any detectable accumulation of YFP:COP1 protein in the nucleus either (Fig. 5C). We conclude that *HY5* levels are positively regulated by low temperature also at the

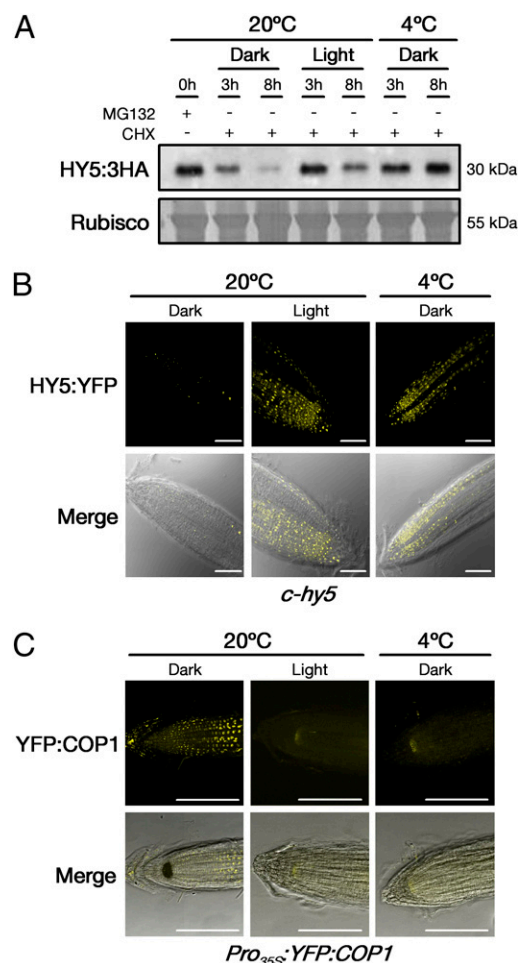


Fig. 5. *HY5* is stabilized in response to low temperature by nuclear depletion of COP1. (A) Levels of *HY5:3HA* protein (30 kDa) in *35S:HY5:3HA*-complemented *hy5-1* seedlings treated with MG132 and subsequently with cycloheximide (CHX), and exposed to 20 °C or 4 °C in the dark or in the light for the indicated times. The large subunit of Rubisco (55 kDa) was used as a loading control. (B) Confocal laser scanning micrographs of *c-hy5* plants exposed to 20 °C or 4 °C in the dark or in the light. (C) Confocal laser scanning micrographs of *cop1-4* mutants complemented with *Pro_{35S}:YFP:COP1* exposed to 20 °C or 4 °C in the dark or in the light. B Lower and C Lower show overlays of the YFP fluorescence and the transmission images. (Scale bars: 75 μm.)

posttranslational level by promoting protein stabilization, which is mediated by nuclear depletion of COP1 as in the case of light signaling.

Discussion

To further understand how cold and light signaling integrate to optimize plant survival under freezing temperatures, we investigated the molecular mechanisms underlying the cold induction of *CAB1*. Our data show that the Z-box, in addition to mediating light-induced gene expression (14), also mediates *CAB1* expression in response to low temperature and constitutes an LTRE. Although several LTREs have been described so far (28–30), to our knowledge the Z-box is the first cis-regulatory sequence reported that integrates light and cold signaling. Light-induced gene expression mediated by the Z-box, including that of *CAB1*, depends on *HY5* (14). Interestingly, our findings reveal that the cold-induced expression of *CAB1* also depends on *HY5*, suggesting a role for this factor in regulating gene expression in response to low temperature. Genome-wide transcriptome analysis

showed that HY5 controls the induction of $\approx 10\%$ of all *Arabidopsis* cold-inducible genes, some of them being related to cold acclimation. These data indicate that HY5 has an important function in regulating cold-induced gene expression and integrates cold and light signaling through the Z-box.

As expected from its role in regulating cold-induced gene expression, our results demonstrate that HY5 acts as a positive regulator of cold acclimation, being required for the full development of this adaptive response. It does not seem, however, to be involved in the constitutive capacity of *Arabidopsis* to tolerate freezing. Consistent with our observation that *hy5-1* mutants accumulate significantly lower anthocyanin levels than WT plants after cold exposure, we proved that HY5 regulates the cold induction of *CHS*, *CHI*, and *FLS* genes, indicating that it activates anthocyanin biosynthesis in response to low temperature. HY5 has also been described to modulate anthocyanin accumulation under light conditions by inducing the expression of *CHS*, *CHI*, and *FLS* (31, 32). Anthocyanins protect photosystems by avoiding the accumulation of high levels of ROS when plants are subjected to different abiotic stresses, including low temperature (24), and consistently we found that *hy5-1* mutants accumulate higher levels of ROS than WT plants in the cold. This increased accumulation of ROS should account for the reduced capacity of *hy5-1* plants to cold acclimate, as it has been suggested for *Arabidopsis* mutants *fro1* (25) and *msr3* (26). The fact that *Arabidopsis* mutants *tt4* and *tt4tt5*, which are defective in anthocyanin biosynthesis and accumulate high levels of ROS (24), are also impaired in cold acclimation, confirms that anthocyanins are needed for a complete cold acclimation response. All these data indicate that HY5 operates in cold acclimation by inducing anthocyanin biosynthesis to restrain ROS accumulation and reveal that HY5 integrates cold and light signaling in the regulation of antioxidant mechanisms. Nonetheless, HY5 should have additional functions in cold acclimation as it regulates the cold induction of different genes related to this adaptive response. The nature of these functions remains to be established.

Our data show that HY5 levels are tightly regulated by low temperature at both transcriptional and posttranscriptional levels, in a similar way as they are in response to light (11, 12). HY5 transcripts rapidly accumulate in response to low temperature independently of the light conditions, this accumulation being regulated at the transcriptional level through an ABA- and CBF-independent pathway. The HY5 promoter does not contain any described LTRE, which suggests that the cold induction of HY5 should be mediated by a new signaling pathway. Posttranslationally, HY5 is stabilized by low temperature. Under control temperature, in the dark, the HY5 protein is turned over in the nucleus by the E3 ubiquitin ligase COP1 (12). In the light, COP1 is inactivated and excluded from the nucleus, allowing HY5 stabilization and activation of light-responsive genes (12). Interestingly, we show that COP1 is excluded from the nucleus also in the dark if *Arabidopsis* is exposed to low temperature, accounting for HY5 stabilization under this adverse environmental condition. Protein stability has also been shown to be important in the regulation of ICE1, a transcription factor having a pivotal role in cold acclimation (33). However, contrary to HY5, in response to low temperature, ICE1 is targeted for proteosomal degradation by the E3 ubiquitin ligase HOS1 in the nucleus, whereas at 20 °C, HOS1 is excluded from the nucleus with the subsequent ICE1 stabilization (34). Therefore, HY5 stabilization by nuclear depletion of COP1 in response to low temperature is not an unspecific inhibitory effect of this abiotic stress on the proteolytic machinery but an accurately regulated process.

Our data support a model (Fig. 6) in which HY5 would promote the development of full cold acclimation, integrating, therefore, low temperature and light signaling. Other light signaling components, including COP1 and the Z-box, would also participate in regulating cold response. In the presence of low temperature, as

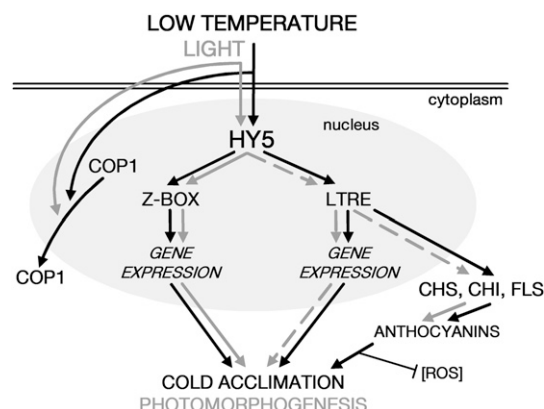


Fig. 6. Proposed model for Z-box, HY5, and COP1 function in cold acclimation. A model in which HY5 would promote full development of cold acclimation, integrating low temperature and light signaling is suggested. The involvement of other light signaling components, including COP1 and the Z-box, is also included. Solid and dotted arrows represent established and theoretical pathways, respectively.

described under light conditions (12), the expression of HY5 would be induced and the nuclear depletion of COP1 would allow HY5 accumulation. Then, paralleling also light signaling (14), HY5 would bind to the Z-box that would act as an LTRE. In addition, consistent with its capacity to promote gene expression by binding to different cis-acting elements (32, 35), HY5 would interact to other still-uncharacterized LTRE motifs. As a consequence, HY5 would activate cold-induced gene expression, ensuring full cold-acclimation development and photomorphogenesis. The cold-induced genes that are activated by HY5 in response to low temperature include those encoding CHI, CHS, and FLS, three key enzymes in the anthocyanin biosynthetic pathway (23). The activation of these genes, which are also light regulated and direct targets of HY5 but do not contain Z-box elements in their promoters (31), would induce anthocyanin biosynthesis restraining ROS accumulation during cold acclimation. As expected from this model, *cop1-4* plants display a low temperature phenotype. They show high levels of anthocyanins as a consequence of accumulating high levels of CHS transcripts (9), which is consistent with their high levels of HY5 and with the function of HY5 as positive regulator of CHS expression. Our findings provide insights on how low temperature and light signals integrate to promote cold acclimation response, and evidence the complexity of the molecular mechanisms plants have evolved to respond and adapt to their fluctuating natural environment.

Materials and Methods

Plant Materials. *Arabidopsis thaliana* (L.) Columbia (Col) and Landsberg *erecta* (Ler) ecotypes, and mutants *hy5-1*, *tt4*, and *tt4tt5* were obtained from the Nottingham Arabidopsis Seeds Centre. The *Arabidopsis* (Col) *CBF1-AS3* transgenic line and the *cbf2-1* mutant were generated in our laboratory (36, 37). *Arabidopsis* (No-0) transformed with *Z:NOS:GUS* and *NOS:GUS* constructs were provided by X. W. Deng (Yale University, New Haven, CT). *hy5-1* mutant plants containing the *Z:NOS:GUS* fusion were obtained from S. Chattopadhyay (National Institute of Plant Genome Research, India). *aba2-11* mutant plants were supplied by P. Rodriguez (IBMCP, Spain). *Arabidopsis* (Ws) transformed with the *Pro_{HY5}:LUC* construct, and mutants *hy5-1* and *cop1-4* complemented with *Pro_{HY5}:HY5:YFP* (*c-hy5*) and *Pro_{35S}:YFP:COP* fusions, respectively, were provided by R. Ulm (University of Geneva, Switzerland). Details on additional materials used in this work are described in *SI Materials and Methods*.

Growth Conditions and Treatments. Growth conditions and treatments for seedlings and plants were essentially as described (38). Details on growth conditions, treatments, and other methods used in this work are described in *SI Materials and Methods*.

Gene Expression Analysis. Expression analyses were performed with 4-d-old etiolated seedlings and 3-wk-old plants by using RNA-blot hybridizations and real-time qPCR. Gene-specific primers are described in Table S2.

Determination of GUS and LUC Activity. GUS activity in 4-d-old etiolated seedlings was detected and measured as described (36). For luminescence imaging, 4-d-old etiolated seedlings were sprayed with 100 μ M luciferin and then kept in the dark for 5 min to avoid fluorescence interference. All images were acquired with 1-min exposure time by using a photon counting I-CCD video camera.

ChIP-qPCR Assays. These assays were performed in 4-wk-old etiolated seedlings as described (39) with some modifications.

Microarray Experiments. For microarray analysis, 3-wk-old *Arabidopsis* Ler and *hy5-1* plants grown at 20 °C were exposed one additional day at 4 °C, and three biological replicates were independently hybridized per transcriptomic comparison. RNA amplification and labeling were carried out as described (40). Hybridization was performed on Agilent *Arabidopsis* Oligo Microarrays v3 in accordance with the manufacturer specifications. Genes with an FDR-corrected *P* value <0.05 and a fold change of more or less than 2 were selected for consideration. Microarray data are deposited in GEO under accession number GSE26314.

Determination of Anthocyanin and ROS Levels. Anthocyanin levels were determined in 2-wk-old plants according to Catala and colleagues (41). ROS accumulation was visualized and quantified in 2-wk-old plants with NBT and DCFH₂-DA, respectively, as described (42).

Immunoblot Analysis. Protein samples (40 μ g) from 10-d-old seedlings were resolved by electrophoresis in 12% SDS-polyacrylamide gels and electrophoretically transferred to a polyvinylidene difluoride membrane according to the manufacturer's protocol. Monoclonal anti-HA was used as primary antibody, and horseradish peroxidase-conjugated anti-rat as secondary antibody. Signal detection was performed as described in the ECL Western detection kit.

Microscopic Analysis. For epifluorescence and light microscopy, 10-d-old seedlings were analyzed with a Confocal Laser Spectral microscope. The excitation line for imaging YFP fusions was 515 nm. Fluorescence was detected in a 560–615 nm band.

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